Deletion of the Met receptor in the collecting duct decreases renal repair following ureteral obstruction

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Hepatocyte growth factor and its receptor, Met, activate biological pathways necessary for repair and regeneration following kidney injury. The Met receptor is expressed in multiple cell types within the kidney, each of which is capable of regulating fibrotic responses. To specifically address the role of the Met receptor in the adult collecting duct during renal injury, a conditional knockout mouse (*Met$^{fl/fl}$;HoxB7-Cre*) was generated and tested using unilateral ureteral obstruction, a model of nephron injury, fibrosis, and repair. Following obstruction in these mice there was increased expression of collagens I and IV along with plasminogen activator inhibitor 1, a known regulator of matrix degradation, compared to ureteral obstructed non-flox littermates. There were trends toward increased interstitial fibrosis, infiltration of the interstitium, and acute tubular necrosis in the knockout mice despite similar degrees of hydronephrosis to the control littermates. The *Met$^{fl/fl}$;HoxB7-Cre* mice; however, had reduced tubular cell proliferation and kidney regenerative capacity after release of the obstruction, thus leading to diminished functional recovery. We suggest that Met receptor signaling in the collecting duct acts as a major regulator of cell survival and propagation of the repair process with a possible secondary role to diminish inflammatory and fibrotic responses.

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The Met receptor and its ligand, Hgf, have been shown to be critical for the formation and maintenance of tubular structures in cells cultured in 3-dimensional matrices through regulation of both cell–matrix and cell–cell interactions.[1–3] Hgf, by binding to its transmembrane receptor Met, a member of the tyrosine kinase superfamily, stimulates an array of downstream signaling pathways resulting in cell proliferation, spreading, migration, as well as inhibition of apoptosis.[4,5] These pleiotropic events have generated much interest for the potential therapeutic role of Hgf in animal models of kidney injury. Recent *in vivo* evidence also supports a role for Hgf as an antifibrogenic factor because injection of the recombinant Hgf protein or gene led to reduced renal myofibroblast activation and less tubulointerstitial fibrosis.[6–9] Conversely, utilizing neutralizing antibodies against Hgf resulted in worsening kidney fibrosis and function in murine chronic kidney injury models.[10] The cell type that is mediating these antifibrotic effects of Hgf is unclear because the Met receptor is expressed in multiple cells including endothelial cells, epithelial cells and some interstitial cells, and each of these are capable of regulating fibrotic responses.

Genetic studies evaluating Met and Hgf in murine models have been limited because the whole animal knockout for either gene results in embryonic lethality.[11,12] To specifically define the role of Hgf–Met signaling in renal fibrosis and matrix accumulation, we have performed collecting duct-specific knockout of Met expression. The collecting duct null *Met$^{fl/fl}$;HoxB7-Cre* mice were found to have normal kidney function although they show a 30% reduction in final nephron number.[13] To investigate the role of the Met receptor in fibrosis and repair we utilized the well-characterized model of unilateral ureteral obstruction (UUO) ± subsequent reversal. Following UUO, our results show that deletion of the Met receptor in the collecting duct resulted in a trend toward worsened interstitial fibrosis, as well as significant increases in acute tubular injury. In addition, upon reversal of the UUO, mice lacking Met receptor expression in the collecting duct showed failure of both histological as well as functional tubule recovery.

RESULTS

Collecting duct-specific deletion of the Met receptor

$Met^{fl/fl}$ mice, in which exon 16 of the *Met* gene is flanked by loxP sites,[14] were mated with *HoxB7-Cre* mice that express the Cre recombinase in the Wolffian duct and ureteric bud-derived structures (Supplementary Figure 1A). Offspring that were heterozygous for the floxed *Met* allele ($Met^{fl/+}$;*HoxB7-Cre*) were mated to generate $Met^{fl/fl}$;*HoxB7-Cre* and $Met^{+/+}$;*HoxB7-Cre* offspring, confirmed by DNA tail genotyping (Supplementary Figure 1B). PCR analysis of DNA from the renal papilla (comprised primarily of collecting duct and thin limbs), revealed that exon 16 of *Met* had been successfully deleted in cells from the renal papilla (Supplementary Figure 1C). Western-blot analysis of renal papilla confirmed that Met protein expression was markedly diminished in this region of the $Met^{fl/fl}$;*HoxB7-Cre* mice (Supplementary Figure 1D), as had been previously reported for Met expression in collecting duct cells.[13]

Unilateral ureteral obstruction (UUO) of $Met^{fl/fl}$;*HoxB7-Cre* mice leads to increased expression of matrix protein genes

To examine the importance of Met signaling in the process of distal nephron injury, fibrosis and repair, a model of unilateral ureteral obstruction was utilized. $Met^{fl/fl}$;*HoxB7-Cre* and $Met^{+/+}$;*HoxB7-Cre* littermates were subjected to UUO of the left ureter for 7 days and the degree of obstructive uropathy analyzed semiquantitatively by the scoring system of Vora *et al.*[15] Compared with the contralateral control kidney, transverse kidney sections from obstructed kidneys of both $Met^{fl/fl}$;*HoxB7-Cre* and $Met^{+/+}$;*HoxB7-Cre* mice showed marked calyceal dilation with no difference in the severity of hydronephrosis between the two groups (Figure 1a, quantitated in Figure 1b).

It is believed that injured tubular cells may undergo de-differentiation to a mesenchymal phenotype and in this state directly promote fibrotic responses. Recently it was shown that treatment with Hgf reduces kidney fibrosis after UUO by diminishing renal interstitial matrix accumulation as well as inhibiting myofibroblast activation.[6] However, it is unclear in these previous studies whether the Hgf is acting directly on the tubular cells, or on nearby endothelial cells, or even at distant sites. To determine whether this antifibrotic response to Hgf is in fact because of decreased expression of pro-fibrotic factors by the injured epithelial cells, UUO was performed in $Met^{fl/fl}$;*HoxB7-Cre* and $Met^{+/+}$;*HoxB7-Cre* littermates for 3 days, followed by isolation of total RNA and quantitative real-time PCR to determine expression levels of factors suggested to mediate the increased fibrosis and matrix accumulation. These experiments showed an increase in the matrix proteins collagen type I, collagen type IV and fibronectin in $Met^{+/+}$;*HoxB7-Cre* mice after UUO (Figure 2a and b), and further revealed that both collagen I and IV were expressed at significantly higher levels in obstructed kidneys from those mice lacking Met expression in the collecting duct. The message for fibronectin in the kidneys of $Met^{fl/fl}$;*HoxB7-Cre* mice was also increased after UUO when compared with control kidneys, although there

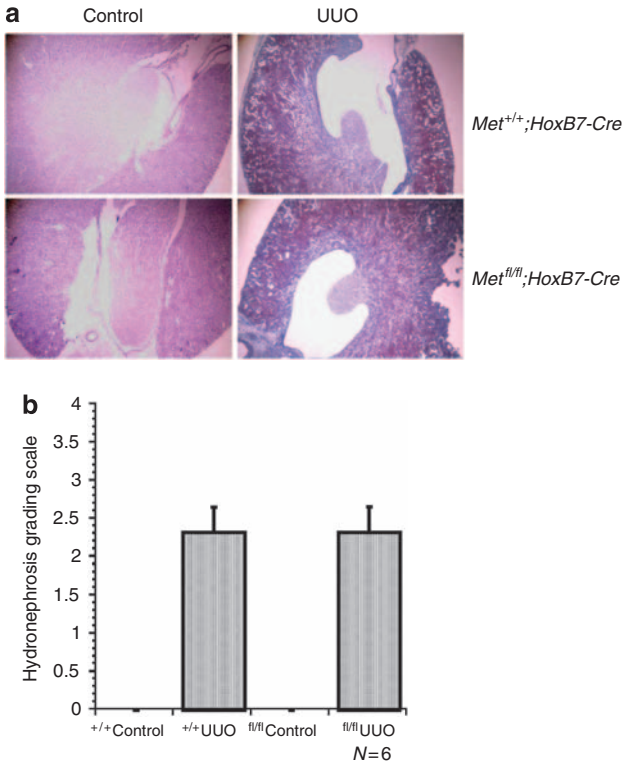

Figure 1 | Semi-quantitative assessment of hydronephrosis. (**a**) Representative Trichrome staining of $Met^{fl/fl}$;*HoxB7-Cre* and $Met^{+/+}$;*HoxB7-Cre* mouse kidney sections (UUO and contralateral control kidneys). (**b**). Quantification of the severity of hydronephrosis from (**a**). $N = 6$. UUO, unilateral ureteral obstruction.

was no statistical difference between the upregulation in obstructed $Met^{fl/fl}$;*HoxB7-Cre* kidneys as compared with obstructed $Met^{+/+}$;*HoxB7-Cre* kidneys (Figure 2c). Consistent with a more fibrotic milieu in the $Met^{fl/fl}$;*HoxB7-Cre* mice, plasminogen activator inhibitor 1, a known inhibitor of matrix degradation, was also significantly increased in the $Met^{fl/fl}$;*HoxB7-Cre* kidneys following UUO (Figure 2d) whereas there was a trend for an increase in transforming growth factor β that did not reach statistical significance (Figure 2e). In contrast, expression of α-smooth muscle actin was unaltered between the two groups (Figure 2f). These data suggest that Hgf/Met signaling normally acts to suppress tubular cell fibrotic responses in a manner that is independent of other markers of cell de-differentiation.

To determine the degree of interstitial fibrosis associated with this increase in matrix protein message expression following UUO-induced hydronephrosis, kidney sections were analyzed on day 1, 3, and 7 following ureteral ligation. The contralateral control kidneys from both $Met^{fl/fl}$;*HoxB7-Cre* and $Met^{+/+}$;*HoxB7-Cre* littermates showed minimal interstitial fibrosis under these conditions (Figure 2g panels 1 and 2, quantitated in 2h). In contrast, trichrome staining from obstructed kidneys showed progressive interstitial fibrosis in both groups, with a trend towards worse fibrosis in the $Met^{fl/fl}$;*HoxB7-Cre* mice, although this did not reach statistical significance.

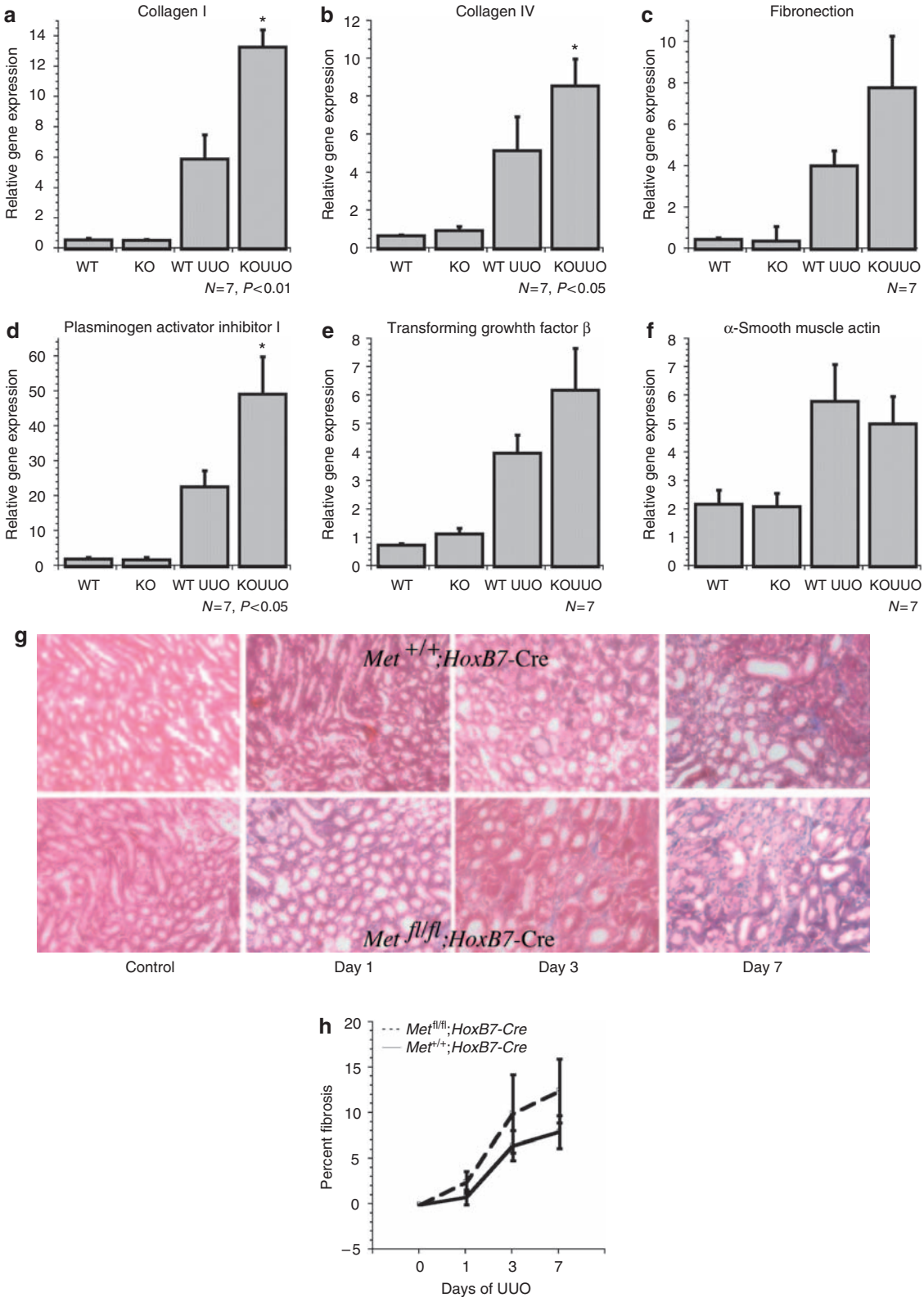

Figure 2 | Quantitative and histological changes in the kidney medulla of *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* mice. (**a**–**f**). Real-time PCR for mRNA levels of the indicated factors in *Met*$^{fl/fl}$*;HoxB7-Cre* kidneys expressed relative to *Met*$^{+/+}$*;HoxB7-Cre* kidneys that have undergone UUO. $N = 7$ for each factor. *$P < 0.01$ vs *Met*$^{+/+}$*;HoxB7-Cre* kidneys for type 1 collagen, *$P < 0.05$ for type 4 collagen, and *$P < 0.05$ for plasminogen activator inhibitor 1. (**g**). Representative Trichrome staining of *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* mice undergoing UUO at time points 1, 3, and 7 days. (**h**) Quantification of percent fibrosis in *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* mice following UUO. $N = 6$. KO, knockout; UUO, unilateral ureteral obstruction; WT, wild type.

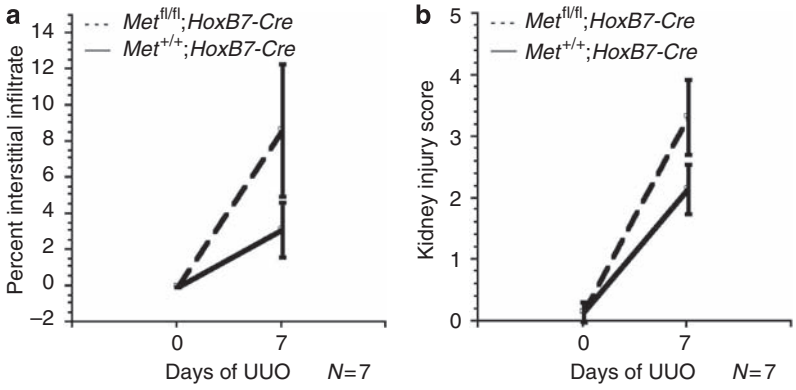

Figure 3 | Comparison of acute tubular injury and interstitial infiltrate in *Met*$^{fl/fl}$;*HoxB7-Cre* kidney vs *Met*$^{+/+}$;*HoxB7-Cre* kidneys following UUO. (**a**) Quantification of percent interstitial infiltrate in *Met*$^{fl/fl}$;*HoxB7-Cre* kidney vs *Met*$^{+/+}$;*HoxB7-Cre* kidney. $N=7$. (**b**) Quantification of acute tubular injury for *Met*$^{fl/fl}$;*HoxB7-Cre* kidney vs *Met*$^{+/+}$;*HoxB7-Cre* kidney. $N=7$. UUO, unilateral ureteral obstruction.

Loss of collecting duct Met expression increases tubular cell apoptosis following UUO

Next, kidney sections obtained from *Met*$^{fl/fl}$;*HoxB7-Cre* and *Met*$^{+/+}$;*HoxB7-Cre* littermates subjected to UUO were evaluated for tubular injury and interstitial infiltrates (as described in the Materials and Methods section). Results showed that at day 7 following UUO there was a marked increase in tubular injury and interstitial cell infiltrates in the UUO kidneys compared with control kidneys, with a trend toward worsened tubular injury ($P=0.052$) and interstitial infiltrate in the *Met*$^{fl/fl}$;*HoxB7-Cre* kidneys (Figure 3a and b), although these differences did not reach statistical significance. Tubular injury secondary to UUO results in increased apoptosis as was shown previously.[16,17] TUNEL stain from kidney sections obtained at time points 1, 3, and 7 days following ureteral ligation of the *Met*$^{fl/fl}$;*HoxB7-Cre* and *Met*$^{+/+}$;*HoxB7-Cre* mice was therefore investigated. As the expression of the Met receptor is specifically deleted in the collecting duct of the *Met*$^{fl/fl}$;*HoxB7-Cre* mice, we assessed the percentage of apoptotic cells that also co-stained with the collecting duct marker, aquaporin-2. The percentage of apoptotic collecting duct cells in the obstructed kidney of the *Met*$^{fl/fl}$;*HoxB7-Cre* mice was significantly increased 1 day following obstruction ($35.6\pm2.6\%$ vs $9.5\pm1.9\%$) ((Figure 4a), quantitated in Figure 4b) compared with the *Met*$^{+/+}$;*HoxB7-Cre* mice. Three days following UUO, a trend toward increased apoptosis in the *Met*$^{fl/fl}$;*HoxB7-Cre* mice collecting duct cells remained compared with the *Met*$^{+/+}$;*HoxB7-Cre* mice, but the difference was no longer statistically significant. Consistent with the finding that the tubular injury score was similar by day 7 in the two groups of mice, the number of tubular cells undergoing apoptosis by this later time point was indistinguishable. Of note, there was also a tendency for increased TUNEL-positive cells in the tubular segments that were not co-stained with aquaporin 2 in the *Met*$^{fl/fl}$;*HoxB7-Cre* mice, but again these did not reach statistical significance.

Reversal of UUO (R-UUO) results in diminished repair in *Met*$^{fl/fl}$;*HoxB7-Cre* mice

It has been recently shown that following release of the ureteral obstruction (R-UUO), a regenerative process occurs within the kidney resulting in histological and functional repair.[18] As numerous groups have showed the importance of Hgf as a tubular cell mitogen and morphogen, inducing proliferation and repair following ischemia reperfusion and cisplatin treatment in rodent models of acute kidney injury,[19–21] we next addressed the importance of tubular cell Met receptor signaling in the repair process following UUO. Using vascular clamps, left ureteral ligation of the *Met*$^{fl/fl}$;*HoxB7-Cre* and *Met*$^{+/+}$;*HoxB7-Cre* mice was performed for 7 days. Compared with the contralateral kidney of *Met*$^{fl/fl}$;*HoxB7-Cre* and *Met*$^{+/+}$;*HoxB7-Cre* mice (Figure 5a upper panel), the ligated kidney in both mouse strains showed the expected findings of interstitial fibrosis, tubular atrophy, and acute tubular necrosis (Figure 5a middle panel). In separate animals, to determine the obstructed kidney's regeneration capacity, the vascular clamps were removed after 7 days of ureteral ligation, and kidneys were allowed to regenerate for 2 weeks. Kidney sections obtained 2 weeks after R-UUO showed a marked decrease in kidney parenchymal restoration in the *Met*$^{fl/fl}$;*HoxB7-Cre* mice, as well as a persistence of cortical thinning and tubular atrophy and fibrosis (Figure 5a bottom panel). Examination of renal mass revealed that sham-operated control kidneys from *Met*$^{+/+}$;*HoxB7-Cre* mice, and *Met*$^{fl/fl}$;*HoxB7-Cre* mice showed a similar weight. Following UUO, kidneys from *Met*$^{+/+}$;*HoxB7-Cre* mice, and *Met*$^{fl/fl}$;*HoxB7-Cre* mice showed a 29 and 34% reduction in weight relative to body weight, respectively (Figure 5b). Following R-UUO in the *Met*$^{+/+}$;*HoxB7-Cre* mice, there was a restoration in kidney weight approaching that seen in unobstructed kidneys. However, R-UUO in the *Met*$^{fl/fl}$;*HoxB7-Cre* mice failed to result in recovery of kidney mass.

Collecting duct proliferation is decreased in *Met*$^{fl/fl}$;*HoxB7-Cre* mice

To address the mechanism for lack of improvement in kidney architecture following R-UUO in the *Met*$^{fl/fl}$;*HoxB7-Cre* mice, we examined tubular cell proliferation in the collecting ducts of *Met*$^{fl/fl}$;*HoxB7-Cre* and *Met*$^{+/+}$;*HoxB7-Cre* mice following R-UUO. Staining with Ki-67 (a proliferation marker) revealed that R-UUO in the kidneys from the

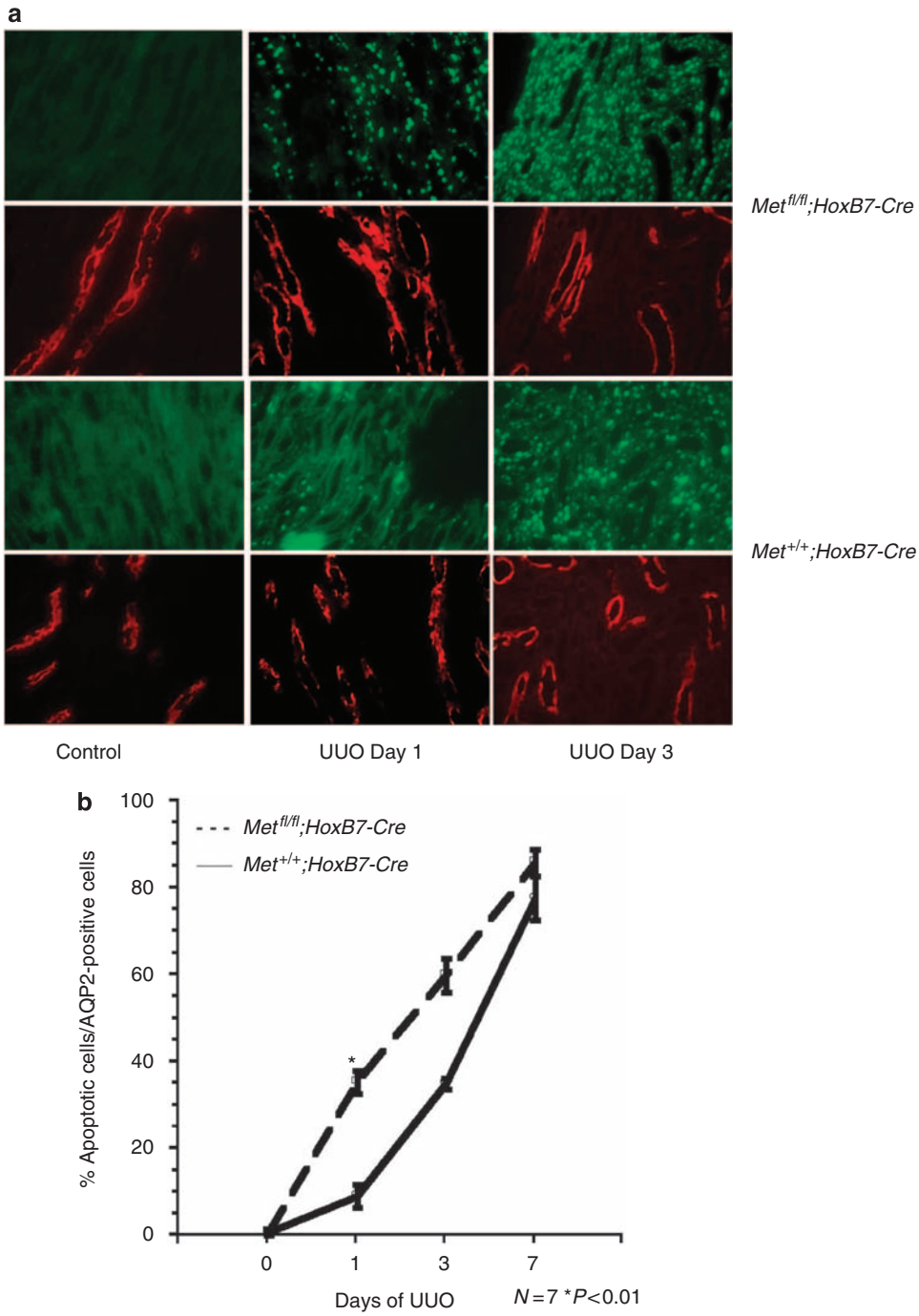

Figure 4 | Comparison of collecting duct apoptosis in *Met*$^{fl/fl}$;*HoxB7-Cre* and *Met*$^{+/+}$;*HoxB7-Cre* mice. (**a**) Representative staining of collecting duct cells for TdT-mediated dUTP nick end labeling (fluorescein isothiocyanate) and aquaporin-2 (Red) following UUO at days 1 and 3 in *Met*$^{fl/fl}$;*HoxB7-Cre* and *Met*$^{+/+}$;*HoxB7-Cre* mice. (**b**) Quantification of the number of apoptotic cells/aquaporin-2(AQP2) positive cells. $N = 7$, $^*P < 0.001$ versus *Met*$^{+/+}$;*HoxB7-Cre* mice at day 1 following UUO. UUO, unilateral ureteral obstruction.

Met$^{fl/fl}$;*HoxB7-Cre* mice resulted in diminished collecting duct tubule proliferation after clamp removal (2 days) when compared with *Met*$^{+/+}$;*HoxB7-Cre* mice (Figure 6a and b). Quantification of the cells co-staining with Ki-67-positive cells and aquaporin 2 showed a significant decrease in collecting duct proliferation ($19.8 \pm 1.9\%$ in *Met*$^{fl/fl}$;*HoxB7-Cre* mice and $73.4 \pm 2.8\%$) in *Met*$^{+/+}$;*HoxB7-Cre* mice (quantitated in Figure 6c). By 2 weeks after R-UUO, the collecting duct proliferation in the *Met*$^{+/+}$;*HoxB7-Cre* mice had diminished to values similar to those in the contralateral unobstructed kidney (data not shown).

To determine the functional impact of accelerated proliferation in wild-type kidneys following R-UUO, the contralateral right ureter was ligated 2 weeks after release of the vascular clamp from the left ureter to restrict GFR to the previously injured left kidney. BUN measurements in these

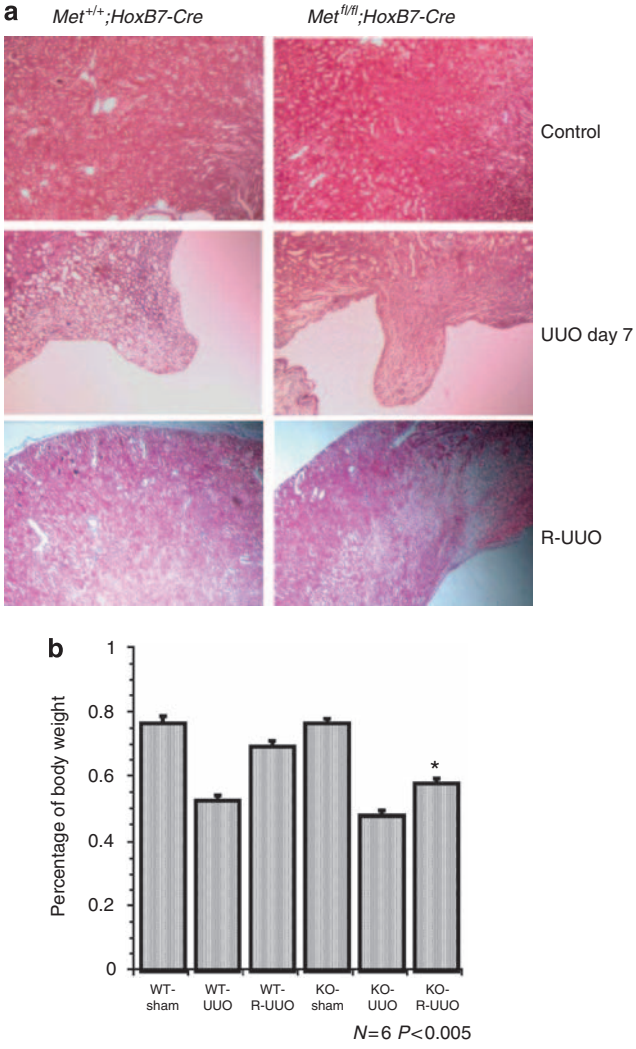

Figure 5 | Histological changes following reversal of UUO in *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* mice. (**a**) Representative Trichrome staining of *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* kidneys (contralateral control kidneys, UUO and reversal of UUO). (**b**) Kidney weight expressed as percentage of body weight in sham-operated mice, UUO, R-UUO $N = 6$ *$P < 0.005$ (Bar 6 compared with Bar 3). R-UUO, reversal of unilateral ureteral obstruction; WT, wild type.

mice 14 days after right kidney ligation showed that R-UUO in the *Met*$^{fl/fl}$*;HoxB7-Cre* mice resulted in a significantly diminished kidney function as compared with *Met*$^{+/+}$*;HoxB7-Cre* mice (74.3 ± 14.6 mg/dl for *Met*$^{fl/fl}$*;HoxB7-Cre* vs 36 ± 2.5 mg/dl for *Met*$^{+/+}$*;HoxB7-Cre* mice) (Figure 6d), suggesting limited improvement in function of the ligated and released left kidney in the mice lacking collecting duct expression of Met. It is interesting to note that the BUN from the *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* mice that underwent conventional UUO with normal contralateral kidneys were indistinguishable.

DISCUSSION

By knocking out Met receptor expression selectively in collecting duct cells, we have begun to dissect the *in vivo* importance of the myriad responses to this growth factor in the process of kidney injury and repair. Hgf stimulation has been shown to protect renal epithelial survival *in vitro* by activating the PI-3 kinase/Akt pathway resulting in phosphorylation and inactivation of pro-apoptotic protein Bad. In addition, recent studies show that Hgf can also induce the antiapoptotic factor Bcl-xL.[22] As apoptosis is the major cause of cell death following UUO, TUNEL assay was performed in our study. Our results show that the loss of Hgf-mediated signaling in the *Met*$^{fl/fl}$*;HoxB7-Cre* mice collecting duct indeed increased apoptosis at 1 day post obstruction. Even after 3 days of obstruction, *Met*$^{fl/fl}$*;HoxB7-Cre* kidneys showed increased apoptosis with a trend toward statistical significance ($P < 0.07$). However, following 7 days of obstruction, apoptosis was extensive in both *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met* $^{+/+}$*;HoxB7-Cre* kidneys, indicating that Met signaling cannot suppress apoptosis if the injury is too severe or prolonged.

In addition to cell survival signaling, Hgf has long been studied because of its ability to induce epithelial cell morphogenic and mitogenic responses that are believed to be important for tubule repair.[23] Cochrane *et al.*, have recently shown that following R-UUO of kidneys from wild-type mice, regeneration takes place during the recovery process and kidney function is partially restored. As it is well documented that Hgf stimulation has a critical role in the recovery from kidney failure in animal models, we compared *Met*$^{+/+}$*;HoxB7-Cre* and *Met*$^{fl/fl}$*;HoxB7-Cre* kidneys following R-UUO. After R-UUO, the destruction of the papilla and outer medulla as well as cortical thinning is repaired progressively and kidney volume is restored from 2 to 6 weeks in the wild-type mice.[18] In our study, the ability of the *Met*$^{fl/fl}$*;HoxB7-Cre* kidney to undergo repair following R-UUO was substantially diminished with marked loss of the proliferative response within the injured tubules in comparison to the *Met*$^{+/+}$*;HoxB7-Cre* kidney. In addition, there was a tendency for these mice to have decreased weight (24.4 g ± 0.29 g) compared with the *Met*$^{+/+}$*;HoxB7-Cre* mice (26.0 ± 0.4 g, $P < 0.06$), suggesting the possibility of decreased intake secondary to uremia. To our knowledge this is the first study to directly show that Met receptor expression specifically in kidney epithelial cells has a critical role in tubular regeneration following injury.

A second major area of interest in the field of renal responses to injury is determining why some episodes of injury lead to fibrosis rather than repair, and how this is normally prevented. In our studies, increased mRNA expression of type 1 collagen, type 4 collagen, and plasminogen activator inhibitor 1 was found in the *Met*$^{fl/fl}$*;HoxB7-Cre* mice following UUO. This is consistent with studies showing that injection of exogenous recombinant human Hgf results in diminished interstitial matrix deposition as well as total collagen accumulation in various rodent models of chronic kidney injury,[24,25] and supports the idea that this antifibrotic response is mediated, at least in part, by the collecting duct cell itself. It has been suggested that Hgf

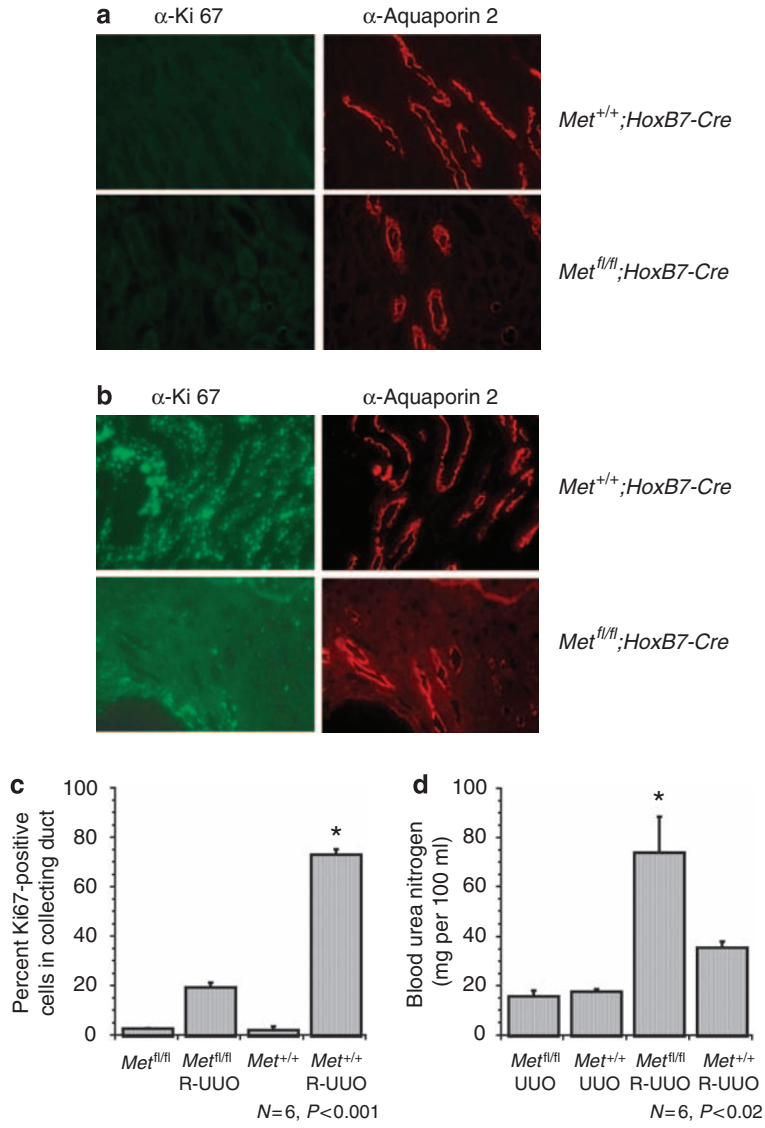

Figure 6 | Comparison of proliferation of collecting duct cells in *Met$^{fl/fl}$;HoxB7-Cre* and *Met$^{+/+}$;HoxB7-Cre* mice following reversal of unilateral ureteral obstruction (R-UUO). (**a**) Representative staining of collecting duct cells with Ki-67 and aquaporin-2 in contralateral control kidneys of *Met$^{fl/fl}$;HoxB7-Cre* and *Met$^{+/+}$;HoxB7-Cre* mice. (**b**) Representative staining of collecting duct cells with Ki-67 and aquaporin-2 following reversal of UUO in *Met$^{fl/fl}$;HoxB7-Cre* and *Met$^{+/+}$;HoxB7-Cre* mice. (**c**) Quantification of percentage Ki-67-positive and aquaporin-positive cells in the collecting ducts of *Met$^{fl/fl}$;HoxB7-Cre* and *Met$^{+/+}$;HoxB7-Cre* mice. $N = 6$, $P < 0.001$. (**d**). Blood urea nitrogen is elevated in *Met$^{fl/fl}$;HoxB7-Cre* mice following reversal of UUO. $N = 6$, $^*P < 0.02$.

exerts its antifibrotic effects by antagonizing Tgf β1-Smad signaling[26] and also by attenuating inflammatory cell infiltration in the kidney by suppressing pro-inflammatory cytokines.[27]

Despite the increased expression of matrix genes by the obstructed *Met$^{fl/fl}$;HoxB7-Cre* kidneys, the loss of Met expression in the collecting duct had no significant effect on fibrosis as judged by scoring of trichrome-stained kidney sections. These results are in contrast with two independent investigators showing that mice injected with neutralizing antibodies to Hgf developed worsened fibrosis in a 5/6th nephrectomy model[8,9,28] and in an ICR strain-derived glomerulonephritis model of CKD.[29] This difference might be because of several factors. First, our studies did show a trend toward worsening fibrosis in the *Met$^{fl/fl}$;HoxB7-Cre* mice, and it may be that with a larger number of experimental animals, a significant difference would be detected. Second, it is possible that the Hgf signaling through several cell types, including endothelial cells and infiltrating inflammatory cells, could act cooperatively to suppress fibrotic responses. In this instance, selective loss of Met expression in the collecting duct cells might not be sufficient to overcome the protective effects induced by the other cell types. Finally, it is possible that the effects reported with administered Hgf in fact reflect a pharmacological response that is not recapitulated by the endogenously produced protein. Resolution of these possible explanations will require

generation of defined Met knockouts in the relevant cell types.

Hence, our study supports the importance of Met receptor signaling in the collecting duct as a major regulator of cell survival and promulgation of the repair process, with a secondary role in diminishing inflammatory and fibrotic responses. These results combined with past studies utilizing systemic Hgf administration suggest that targeting Hgf activation to the collecting duct may provide a promising novel approach for minimizing acute tubular injury and promoting effective repair.

MATERIALS AND METHODS

Antibodies

Antiaquaporin-2 and Met antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-Ki67 was purchased from Novus Biologicals (Littleton, CO, USA), and anti-E-cadherin antibody from BD Biosciences (Franklin Lakes, NJ, USA).

Creation and genotyping of conditional Met knockout mice

The $Met^{fl/fl}$ mouse was developed on the 129SV/C57Bl/6 background as described.[14] *HoxB7-Cre* mice on the C57Bl/6 background were purchased from Jackson Laboratory, Bar Harbor, ME, USA. Tail genotyping was performed using the Met forward primer (F) 5′-ttag gcaatgaggtgtcccac-3′ and reverse primer (R) 5′-ccaggtggcttcaaattcta agg-3′. To detect deletion of Met in the collecting duct, primer 5′-cagccgtcagacaattggcac-3′ and primer 5′-ccaggtggcttcaaattctaagg-3′ were used. The expected sizes of wild-type allele, floxed allele, and deleted allele were 380, 300, and 650 bp, respectively. To determine Cre expression, CreF 5′-ccgggctgccacgaccaa-3′ and CreR 5′-ggcgc ggcaacaacattttt-3′ primers were used, generating a 400-bp fragment. In all experiments, homozygous littermates ($Met^{fl/fl}$;*HoxB7*-Cre and $Met^{+/+}$;*HoxB7*-Cre mice) generated from the same breeding pair ($Met^{fl/+}$;*HoxB7*-Cre mice) were used as experimental subjects and their respective controls. All mouse experiments were performed under a protocol approved by the Yale IACUC.

Protein isolation and western blot analysis

Mice were anesthetized by intraperitoneal injection of Ketamine (100 mg/kg) and Xylazine (20 mg/kg), kidneys extracted and renal papilla surgically removed. The papilla was homogenized in saline with EDTA-free protease inhibitor (Complete, Roche, Indianapolis, IN, USA) using a Dounce homogenizer and centrifuged at 100,000 *g*. The pellet was resuspended in 100 µl of PBS and 40 µg of protein/sample separated by SDS-PAGE, electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA), immunoblotted with the appropriate antibody and visualized by enhanced chemiluminescence (ECL, Amersham Biosciences, Pittsburgh, PA, USA).

Kidney immunofluorescence and histology

Mice were anesthetized by intraperitoneal injection of Ketamine and Xylazine followed by perfusion fixation with 40 ml of 4% PFA. The kidneys were frozen, sectioned at 4 µm, and subjected to antigen retrieval (Retrievagen, BD Franklin Lakes, NJ, USA) followed by blocking with 1% bovine serum albumin for 1 h. Immunostaining was performed with the appropriate primary antibody overnight at 4°C, followed by Alexa Fluor donkey anti-rabbit 488 or donkey anti-goat 594 secondary antibodies (Invitrogen, Carlsbad, CA, USA) for visualization. 4,6,-diamidino-2-phenylindole (DAPI) was included in the mounting medium as a counterstain (Vector Laboratories, Burlingame, CA, USA). For kidney histology, mice were perfusion fixed as above followed by H&E or Trichrome staining performed by the Yale Pathology Department. For the TUNEL assay, FITC-conjugated TUNEL enzyme (Roche) was used as per manufacturer protocols and expressed as the percentage of aquaporin 2-positive cells that was also TUNEL positive.

Unilateral ureteral obstruction and reverse (UUO and R-UUO)

Mice were placed under anesthesia with Xylazine and Ketamine. UUO was performed on 8-week-old male $Met^{fl/fl}$;*HoxB7-Cre* and $Met^{+/+}$;*HoxB7-Cre* littermates (derived from mating $Met^{fl/+}$;*HoxB7-Cre* mice) by ligation of the left ureter of each animal at the ureteropelvic junction using 4.0 silk through a flank incision. After 1, 3, or 7 days following the procedure, the animals underwent perfusion fixation with 4% paraformaldehyde in PBS. Unobstructed kidneys were used as controls and histology obtained. These studies were performed in an accredited animal care facility in accordance with the National Institutes of Health (NIH) 'Guide for the Care and Use of Laboratory Animals.' For UUO reverse (R-UUO), identical flank incision was performed and left ureter ligated by vascular clamp (0.4 mm; Fine Science Tools, Foster City, CA, USA). Mice were clamped for 7 days at which time the clamp was released under isofluorane anesthesia and kidneys allowed to recover for 2 weeks. A separate group of mice underwent right ureter ligation with 4.0 silk at the time of release of the left kidney clamp, with BUN and histology obtained at 2 weeks after left kidney recovery.

Morphometric evaluation of interstitial fibrosis and kidney injury

Histological examinations were performed in a single-blinded manner by a renal pathologist (GM). Histological changes were quantified by calculation of the percentage of tubules that displayed cell necrosis, loss of brush border, cast formation, and tubule dilation as follows: 0, none; 1, 10%; 2, 11–25%; 3, 26–45%; 4, 46–75%; and 5, $\geqslant$76%. At least 10 fields ($\times$200) were reviewed for each slide.[30] Morphometry of renal cortex (interstitial fibrosis, and interstitial infiltration defined by neutrophils, plasma cells, and eosinophils) was performed using a point counting technique. Slides were examined on a $\times$10 objective. Images were captured by an Optronics camera and projected onto a monitor overlaid with a point grid. Points falling on tubules; interstitial edema/inflammation/dilated capillaries; interstitial fibrosis; and vessels were counted, and the percentage of cortex with infiltrate and fibrosis was expressed as percentage of the total area evaluated.[31] One-way ANOVA using Kruskal–Wallis test and *t*-test were used for statistical analysis.

Morphometric evaluation of hydronephrosis

The degree of hydronephrosis was determined in a blinded fashion using the criteria of Vora *et al.*[15] A grading scale from 0 to 3 was used with 0 = normal (finger-in-glove configuration of the papilla and calyx); 1 = minimal (a narrow but definable fluid-filled calyceal space with normal papillary contour); 2 = moderate (unequivocal dilatation of the calyx with compression of the papilla, but the preservation of its conical shape); 3 = marked (gross distension of the calyx, typically increasing overall volume of the kidney by at least 50% and resulting in severe compression of the lateral cortex and distortion of the papilla).

Quantitative PCR

Kidneys from 8-week-old *Met*$^{fl/fl}$*;HoxB7-Cre* and *Met*$^{+/+}$*;HoxB7-Cre* littermates that underwent UUO were harvested and total RNA isolated using the RNeasy kit (Qiagen Valencia, CA, USA). One microgram of RNA was reverse transcribed using random hexamer primers (SuperScript II, Invitrogen) and qPCR was conducted using power SYBR green mix (Applied Biosystems, Foster City, CA, USA) with a 7300 AB real-time PCR machine (Applied Biosystems). The primers used for PCR were selected for an efficiency of 90–100% and are included in the Supplementary Table. Results for each factor were normalized to Gapdh expression from the same PCR (expressed as dCt).

Clinical chemistry and statistics

Plasma and urine electrolytes were analyzed using the Yale University School of Medicine Core Mouse Metabolic Phenotyping Center. The data is expressed as mean ± s.e.m. Statistical significance was determined using Student's *t*-test.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Collecting duct knockout of Met expression
Table. Primer Table
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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